MULTIPLE MOLEKULARE FORMEN DER ALKOHOLDEHYDROGENASE AUS PFERDELEBER

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J.P. von Wartburg, P.M. Kopp, U.M. Lutstorf
Properties of Horse Liver Alcohol Dehydrogenase Isoenzymes
FEBS Symposium, Vol. 18, 195–202 (1970)

U.M. Lutstorf, J.P. von Wartburg
Subunit Composition of Horse Liver Alcohol Dehydrogenase Isoenzymes
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U.M. Lutstorf, P.M. Kopp, J.P. von Wartburg
Heterogeneity of Horse Liver Alcohol Dehydrogenase Purification
and Characterization of Multiple Molecular Forms
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Properties of Horse Liver Alcohol Dehydrogenase Isoenzymes

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Heterogeneity of horse liver alcohol dehydrogenase was first reported by Dalziel [1], who separated crystalline enzyme preparations into two distinct fractions by electrophoresis and chromatography. Three isoenzymes are found in rhesus monkey [2]. Two of them occur in the liver [3]; the third is localized mainly in the gastro-intestinal tract. The two isoenzyme preparations isolated from liver show marked differences in their catalytic properties [3]. Five fractions of ADH could be detected in horse liver by means of electrophoresis on agar gel [3, 4]. Similar electrophoretic patterns were obtained by McKinley-McKee and Moss on starch gel [5]. An improved chromatographic procedure permits the separation of up to seven different fractions [6, 7].

Previous studies have revealed two identical active sites in horse [8] and human liver alcohol dehydrogenase [9], suggesting a dimeric structure of these enzymes. If two non-identical subunits exist, their random aggregation would result in the formation of three active isoenzymes. This is the number of isoenzymes actually found in rhesus monkey and man [6, 10]. The existence of more than three fractions of ADH in horse liver might be expected on the basis of a tetrameric structure of the enzyme in this particular species. Four subunits in horse liver ADH have been suggested by Vallee and his collaborators [11]. Evidence for only two subunits arises from studies on the primary structure of the main fraction of horse liver ADH carried out by Jörnvall and Harris [12]. The results presented in this paper are consistent with a dimeric structure of ADH isoenzymes in horse liver. The separation and purification of the isoenzymes of horse liver ADH was achieved by chromatography on carbo-

Non-Standard Abbreviations: Alcohol dehydrogenase, ADH; gram atoms, gm. at.; molecular weight, mol. wt.; isoelectric point, IEP; 5-dihydrotestosterone, DHT; phenylethanediol, PED.

Enzymes. Alcohol dehydrogenase or alcohol: NAD oxidoreductase, EC 1.1.1.1; Peroxidase or donor: hydrogenperoxide oxidoreductase, EC 1.11.1.7.

xymethyl- and diethylaminoethyl-cellulose [7]. Three fractions (IIc, III and IV) have been crystallized from methanol-containing buffer. The electrophoretic mobility on agar gel of the purified isoenzymes is shown in Fig. 1. The three fractions IIa, IIb and IIc move close to each other, so that no complete electrophoretic separation can be obtained on crude horse liver extracts. Larger differences exist, however, between the electrophoretic mobilities of isoenzymes III, IV and V. The model of a tetramer built by random combination of two polypeptide chains with unequal charge would predict five fractions with corresponding differences in electrophoretic mobility. The existence of seven distinct chromatographic fractions with unequal distances between the electrophoretic bands can only be explained on the basis of a more complex model.

One possibility is the existence of active protomers with molecular weights of 40,000 or 160,000. The molecular weight of the main fraction (isoenzyme III) is known to be approximately 80,000 [11, 13]. The molecular weights of the purified ADH fractions were estimated by gel filtration on a Sephadex G-200 column (Table 1). The values for the elution volumes of all fractions are nearly identical, and distinct from those obtained from horse radish peroxidase (EC 1.11.1.7, mol. wt. 44,000 [14]) and yeast alcohol dehydrogenase (EC 1.1.1.1, mol. wt. 150,000 [15]). The liver and yeast enzymes are known to be

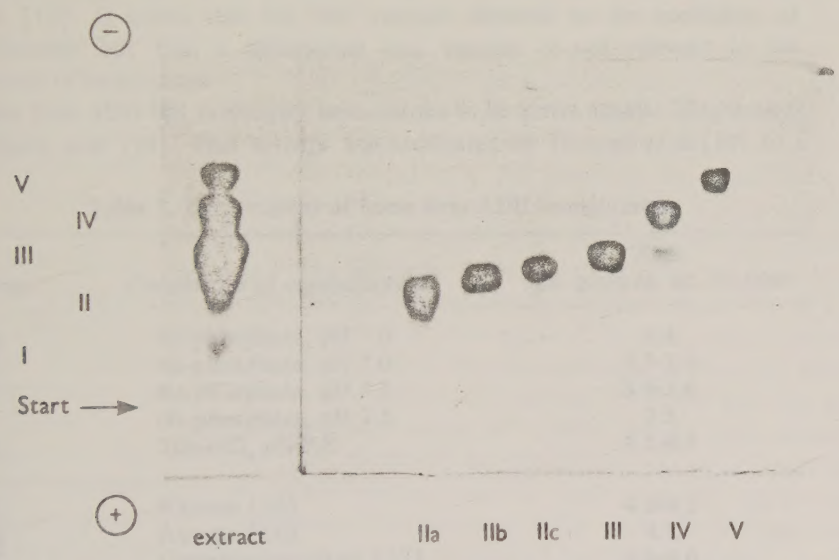

Figure 1. Agar gel electrophoresis of horse liver ADH isoenzymes. Electrophoresis at pH 9.0, Tris-HCl buffer [3]. Staining for ADH-activity by incubation with nitrotetrazolium blue 0.42 mg/ml, phenazine metasulfate 0.034 mg/ml, ethanol 2.5×10^{-2}M, NAD 1.25×10^{-3}M at 37°C.
Left: crude liver extract; right: fractions separated and purified by chromotography.

Table 1. Gel filtration of horse liver ADH isoenzymes.

	Horse liver alcohol dehydrogenase							
Enzyme	IIa	IIb	IIc	III	IV	V	Peroxidase	Yeast ADH
v_e/v_o	1.82	1.94	1.89	1.82	1.90	1.90	2.20	1.61

Mean value of two determinations of v_e/v_o on a Sephadex G-200 column (1.9 x 27.5 cm); flow rate 12 ml/h; fraction volume 2.0 ml; Na-phosphate, pH 7.0, 0.05M; sample, 2 mg protein in 2 ml buffer; determination of v_o with blue dextran; 4°C.

metallo-enzymes. It is conceivable that a variable zinc content could lead to different electrophoretic mobilities. In their purest enzyme preparations, Vallee and his collaborators [11] found 3.4-3.6 gm. at. of zinc per mol. wt. 80,000. Values of 3.8-4.2 gm. at. per mol. wt. 84,000 were found in Theorell's laboratory [16] and by Oppenheimer *et al.* [17]. The zinc content of the four fractions IIc, III, IV and V of our preparations ranged from 3.3-3.6 gm. at. per mol. wt. 80,000 (Table 2) when the enzyme is crystallized at pH 7.0-7.5. However, crystallization at pH 8.8 leads to the incorporation of 4 gm. at. of zinc. An identical zinc content of fractions IIc (the minor fraction in Dalziel's preparation [1]) and III is in accord with the determinations reported by Åkeson [16]. It seems that the zinc content depends on the conditions of crystallization but that a differential zinc content is not relevant to the occurrence of isoenzymes.

Horse liver ADH has previously been shown to be active against 3β-hydroxy-5β-cholanic acid [18]. This activity was attributed by Theorell *et al.* [19] to a

Table 2. Zinc content of horse liver ADH isoenzymes.

Enzyme	Conditions of crystallization	Zinc gm. at./mol. wt. 80,000
IIc	Na-phosphate, pH 7.0	3.4
III	Na-phosphate, pH 7.0	3.3-3.6
IV	Na-phosphate, pH 7.5	3.4-3.6
V	Na-phosphate, pH 7.5	3.5
III	Tris-HCl, pH 8.8	4.1-4.3
III	Åkeson [16]	4.0-4.2
IIc	Åkeson [16]	4.2
III	Oppenheimer *et al.* [17]	3.8-4.0
III	Drum *et al.* [11]	3.4-3.6

Zinc determinations by atomic absorption (Unicam No. SP 90) of enzyme solutions dialyzed against metal free buffer [9].

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particular enzyme, the steroid-active liver alcohol dehydrogenase. This subfraction is more basic than the main fraction and corresponds to isoenzyme IV in our preparations. The amino acid composition of both fractions was found to be nearly identical. Therefore it was considered by these authors that the difference in electrophoretic mobility might be due to different amide nitrogen contents. According to our determinations, isoenzymes III, IV and V all contain 28 ± 1 acid amide residues (Table 3).

Table 3. Acid amide content* of horse liver ADH isoenzymes.

Enzyme	III	IV	V
Mean value	28.1	28.5	27.1
1st determination	27.0	29.3	27.8
2nd determination	29.2	27.6	26.5

*Acid amide groups per mol. wt. 80,000, determined according to Knight and Blomstrom [27].

Various buffer ions are known to influence the electrophoretic mobility of proteins. The IEP of pH 6.8 for the main fraction of horse liver ADH (isoenzyme III) was determined by Dalziel [1] by free boundary electrophoresis in phosphate buffer. When the pronounced endosmosis in agar gel is taken into account, isoenzyme III shows only a small anodic mobility at pH 9.0 (Fig. 1), indicating that the IEP might be higher than pH 6.8 in Tris buffer. Isoenzyme IV migrates slowly to the cathode, suggesting an IEP above pH 9.0. Therefore, the isoelectric points of both isoenzymes were determined by the method of isoelectric focusing (Electrofocusing column LKB 8101 and ampholytes from LKB-Produkter AB, Stockholm-Bromma 1, Sweden) according to Vesterberg and Svensson [20]. In this system, which is free of buffer ions, an IEP of 8.7 was found for isoenzyme III and 9.3 for isoenzyme IV. This result demonstrates that the binding of buffer ions can alter the electrophoretic mobility of ADH. Nonetheless, the possibility of a differential binding of buffer ions can be excluded as a cause for the occurrence of ADH fractions with distinct electrophoretic mobilities. The separation of isoenzymes can be achieved by zone electrophoresis on several carriers, such as polyacrylamide, starch and cellulose acetate in the presence of a variety of buffer ions. Furthermore, the electrophoretic mobility of isoenzymes is unaffected during purification.

Ursprung and Sofer [21] have shown that new electrophoretic bands arise after the preincubation of purified ADH from Drosophila melanogaster in the presence of NAD. They are due to the formation of non-specific enzyme-coenzyme complexes. The extent of this conversion depends on time and

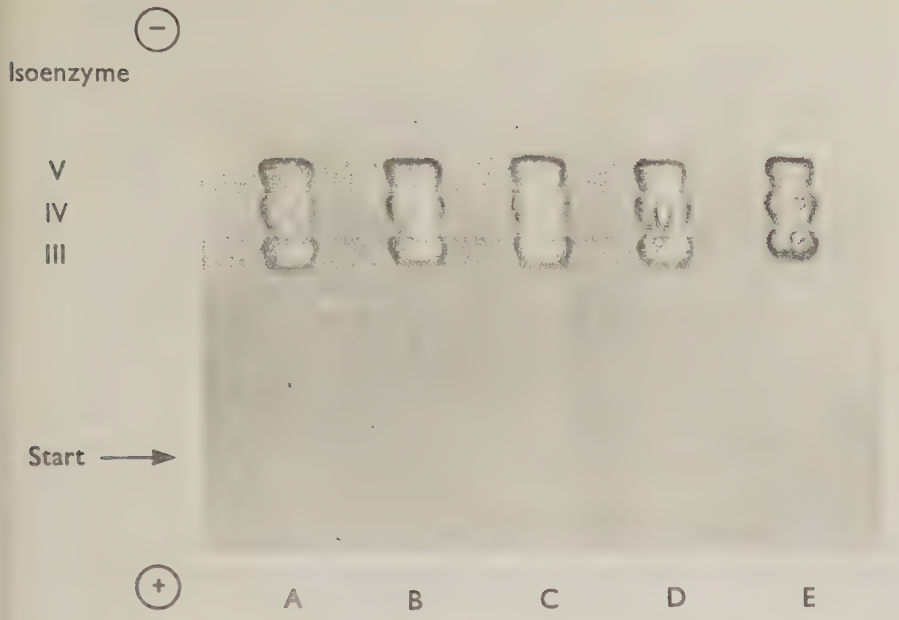

Figure 2. Electrophoretic pattern of horse liver ADH isoenzymes after preincubation with coenzymes. For the electrophoretic procedure see Fig. 1. Conditions of incubation: control without coenzyme (A), 1.0×10^{-2}M NAD (B), 1.0×10^{-3}M NAD (C), 1.0×10^{-2}M NADH (D), 1.0×10^{-3}M NADH (E), in Na-pyrophosphate pH 8.8, 2×10^{-2}M at 4°C for 2 h. Identical patterns were obtained after 24 and 48 h incubation.

coenzyme concentration. McKinley-McKee and Moss [5] previously proposed a similar mechanism in order to explain the heterogeneity of horse liver ADH. We therefore incubated a mixture of pure isoenzymes III, IV and V with coenzyme in the oxidized and reduced form. No shift in the position of the activity of the single isoenzymes, and no formation of new bands at the localization of isoenzymes IIa, b and c, could be observed (Fig. 2).

Structural interrelations between isoenzymes may also be reflected in the catalytic properties, provided that the enzymatic function of the single polypeptide chains is not grossly changed when combined to a protomer. Therefore, some catalytic parameters of the purified ADH fractions were determined. Pyrazole has been shown by Theorell and Yonetani [22] to be an inhibitor of ADH, which is competitive with the substrate ethanol. According to Vallee *et al.* [23], *o*-phenanthroline inhibits ADH by chelating the functional zinc, thus competing with the coenzyme. No marked differences are found between fractions I, IIa, b and c in respect to the inhibitor concentrations needed for a 50% inhibition of ADH-activity (Table 4). Increasing concentrations are needed for both inhibitors to achieve the same effect with isoenzymes III, IV and V.

Table 4. Inhibition of horse liver ADH isoenzymes by pyrazole and *o*-phenanthroline.

Isoenzyme	I	IIa	IIb	IIc	III	IV	V
Pyrazole	0.4	0.6	0.4	0.7	35	73	125
o-Phenanthroline	170	250	320	190	310	820	9000

Inhibitor concentrations (μM) required for 50% inhibition (I_{50}). Conditions: Na-pyrophosphate-buffer, pH 8.8, 3.3×10^{-2}M; NAD, 1.6×10^{-3}M; ethanol, 1.6×10^{-2}M; initial velocity measurements at 25°C.

According to Theorell *et al.* [19] isoenzyme IV is, at least in part, responsible for the steroid activity of horse liver ADH. By partial separation of the five electrophoretic fractions, Pietruszko *et al.* [24] demonstrated that the fraction with the most basic character exerts even a higher steroid activity, whereas the two more acidic fractions and the main fraction show only little activity with 5β-androstan-17β-ol-3-one (5-dihydrotestosterone, DHT). The results of the determination of the activity ratios of DHT to acetaldehyde reduction for pure fractions IIa, IIb, IIc, III, IV and V are listed in Table 5. No activity with DHT was detectable for isoenzyme III, thus confirming the observation made by Theorell *et al.* [19]. Isoenzyme IV reduces DHT at a rate which is 50 times lower than the one for acetaldehyde. For isoenzyme V the rate with the steroid is almost half that with acetaldehyde. Analogous results are also obtained with phenylethanediol as a substrate. It seems that the same steric requirements as for 3β-hydroxy of 3-keto-steroids must be fulfilled for this compound. In this context, it is noteworthy that Winer [25] observed 2-phenylethanol to be a good substrate for the main fraction of horse liver ADH (isoenzyme III).

Table 5. Enzymatic activities of horse liver ADH isoenzymes in the presence of 5-dihydrotestosterone and phenylethanediol.

Isoenzyme	$\frac{V_{AcAld}}{V_{DHT}}$	$\frac{V_{EtOH}}{V_{PED}}$
IIa	n.d.	n.d.
IIb	n.d.	n.d.
IIc	n.d.	n.d.
III	n.d.	n.d.
IV	50	30
V	2.3	2.1

Conditions: Na-phosphate buffer, pH 7.0, 3.3×10^{-2}M; NADH, 3.3×10^{-4}M; acetaldehyde (AcAld), 1.0×10^{-3}M; 5-dihydrotestosterone (DHT), 3.3×10^{-4}M; Na-pyrophosphate-buffer, pH 8.8, 3.3×10^{-2}M; NAD, 1.6×10^{-3}M; ethanol (EtOH), 1.0×10^{-3}M; phenylethanediol (PED), 1.0×10^{-3}M.
n.d. = no detectable activity with DHT or PED.

These results indicate that isoenzyme V, as would be expected from the structural model, is much more active as a steroid dehydrogenase than isoenzyme IV. They also suggest, together with the inhibition studies, that the group of fractions IIa, b and c is distinct from isoenzymes III, IV and V. It is probable that isoenzymes III, IV and V correspond to the structural models AA, AB and BB. Monomerization and reactivation of isoenzyme III under the conditions described by Drum *et al.* [11] yield only one enzyme with an electrophoretic mobility identical with that of the original material. An analogous result is obtained with isoenzyme V. If isoenzyme IV is treated in the same way, a mixture of isoenzymes III, IV and V is formed.

All three isoenzymes are also found after monomerization and reactivation of a mixture of pure isoenzymes III and V, representing an *in vitro* hybridization. No enzymatic fractions corresponding to fractions IIa, b or c were formed in either of the experiments. These hybridization studies [26] are consistent with the proposed model for isoenzymes III, IV and V. Together with the investigation on the catalytic parameters, they provide evidence in support of a dimeric structure for the isoenzymes of horse liver alcohol dehydrogenase. The possibility that the occurrence of the fractions I, IIa, IIb and IIc is due to factors other than genetic determination cannot be ruled out.

ACKNOWLEDGEMENTS

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SUBUNIT COMPOSITION OF HORSE LIVER ALCOHOL DEHYDROGENASE ISOENZYMES*

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1. Introduction

During the last few years several protomeric enzymes have been shown to exist in multiple molecular forms produced by random combination of non-identical subunits. Three isoenzymes of alcohol dehydrogenase (ADH; E.C. 1.1.1.1) are found in rhesus monkey and man [1–3] indicating that ADH is a dimer composed of two different subunits. Recently a dimeric structure has been reported for the main fraction of alcohol dehydrogenase from horse liver [4,5]. However, in this species more than three enzymatically active fractions can be distinguished by electrophoresis and chromatography [6,7]. Starch gel electrophoresis reveals up to nine fractions [8,9]. The present communication reports on the subunit composition of these horse liver ADH fractions.

2. Experimental

2.1. *Electrophoresis*

The method for the agar gel electrophoresis has been described previously [1]. Starch gels were prepared according to Smithies [10]; gel buffer, Tris-HCl 0.025 M, pH 8.5; vessel buffer, Tris-HCl 0.3 M, pH 8.5. The electrophoresis was carried out with 10 V/cm during 5 hr at 4°C. ADH bands were visualized by activity staining [3].

* Part of this work has been presented at the 6th FEBS Meeting in Madrid (1969).

2.2. *Monomerization and hybridization*

Reversible dissociation was performed under conditions described by Drum et al. [11]. Incubation of ADH with 8 M urea and 0.1 M mercaptoethanol in 0.1 M sodium phosphate buffer pH 7.5 at 21°C led to a complete loss of activity within 10 min. The activity could partially be restored by dilution with 10 volumes of the same buffer containing 6×10^{-6} M zinc chloride and 6×10^{-4} M NADH and subsequent incubation for 2 hr at 21°C. The reactivation mixture was dialyzed against 100 volumes of 0.1 M sodium phosphate buffer pH 7.5 for 14 hr at 4°C prior to activity measurements and electrophoresis.

3. Results and discussion

As shown in fig. 1, the electrophoresis of crude horse liver extracts on agar gel reveals five bands as previously reported [2,6,12,13]. The higher resolution provided by starch gel makes it possible to demonstrate that the broad band II on agar gel consists of three distinct subbands (IIa, IIb and IIc). They can be separated on a preparative scale by chromatography on DEAE cellulose. Starch gel electrophoresis of crude liver extracts reveals two additional bands between isoenzyme III and IV which we are naming IVa and IVb. Similar electrophoretic patterns showing up to nine fractions have recently been described by Pietruszko et al. [8].

Based on kinetic studies of substrate specificity or effects of inhibitors and some hybridization experiments we have suggested a subunit composition AA, AB and BB for isoenzyme III, IV and V respectively [7]. The results of monomerization and reactivation

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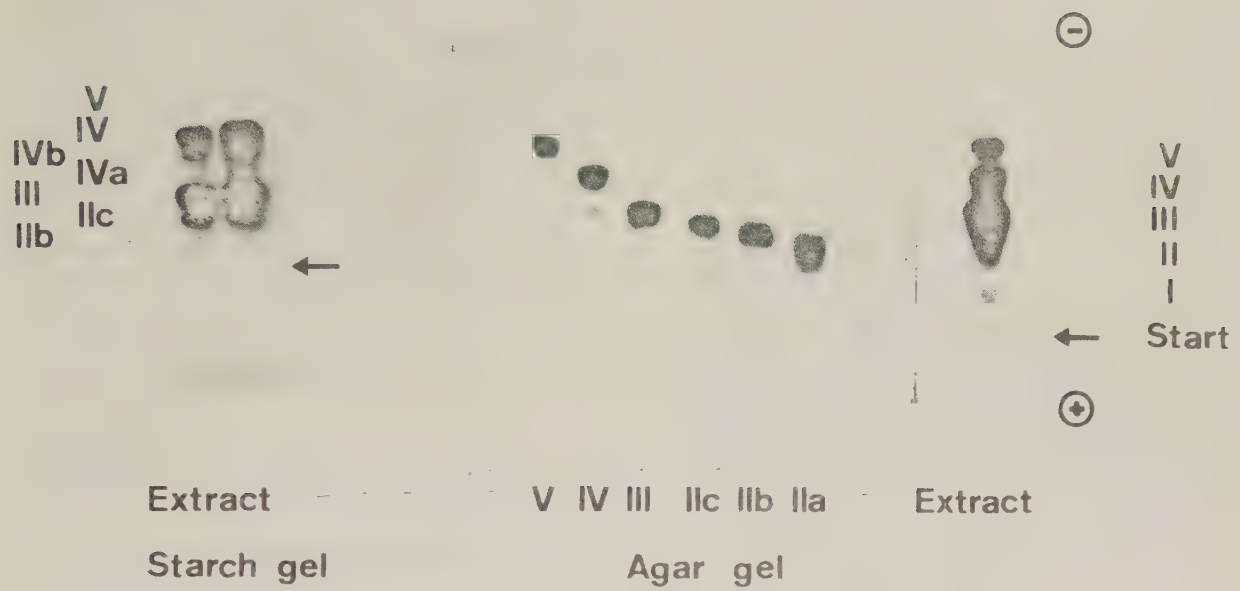

Fig. 1. Electrophoresis of horse liver ADH. Electrophoretic pattern of crude extract on starch gel at pH 8.5, Tris-HCl buffer (left) and on agar gel at pH 9.0, Tris-HCl buffer (right). Electrophoresis on agar gel at pH 9.0, Tris-HCl buffer of fractions separated and purified on ion exchange celluloses (middle). ADH bands are visualized by activity staining.

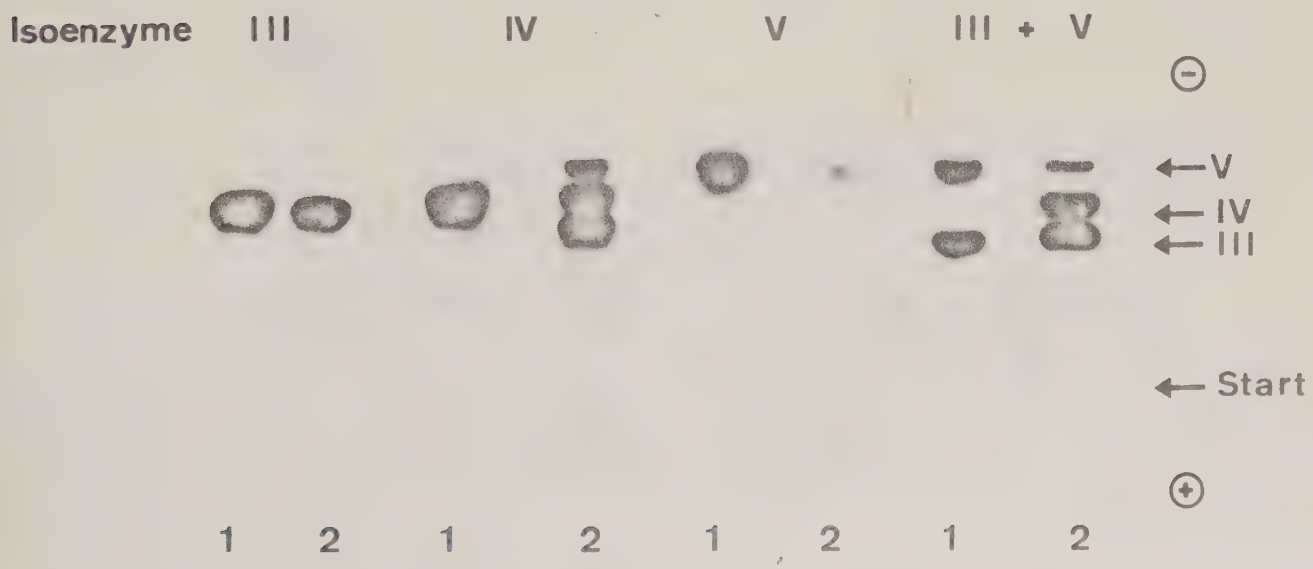

Fig. 2. Hybridization of horse liver ADH isoenzymes. Electrophoresis on agar gel at pH 9.0, Tris-HCl buffer was carried out before monomerization [1] and after reactivation [2]. For experimental procedure see text.

of pure isoenzyme III, IV and V and of the hybridization of a mixture of III and V are shown in fig. 2. As expected, isoenzyme III and V behave like enzymes containing identical subunits. The high degree of monomerization is exemplified by the extensive formation of the hybrid enzyme during reversible inactivation of a mixture of isoenzyme III and V. In agreement with these findings the hybrid enzyme IV yields all three isoenzymes upon monomerization and subsequent recombination. None of the other electrophoretic fractions known to exist in crude horse liver extracts are formed in any of these experiments. These results are in accordance with the observations recently published by Pietruszko and Theorell [14].

Crystals of fractions IIc and III obtained from 5% (v/v) methanol in phosphate buffer are shown in fig. 3. If the repeatedly washed crystals of isoenzyme III are redissolved and submitted to electrophoresis, pure isoenzyme III is found. In the supernatant, however, isoenzyme III partly converts to fraction IIc (fig. 3a); the amount of IIc increases with the time of storage. An analogous conversion of fraction IIc to isoenzyme III can be observed (fig. 3b). The conversion of isoenzyme III to fraction IIc is a slow process, requiring weeks for the formation of considerable amounts of IIc. The rate of conversion is not enhanced under the conditions of monomerization and reactivation. On the other hand, the reverse process, that is the conversion of fraction IIc to isoenzyme III, occurs at a faster rate and is accelerated by monomerization. As shown in fig. 4a, isoenzyme III is formed to a large extent during the 20 hr required for monomerization, reactivation and dialysis of fraction IIc. Isoen-

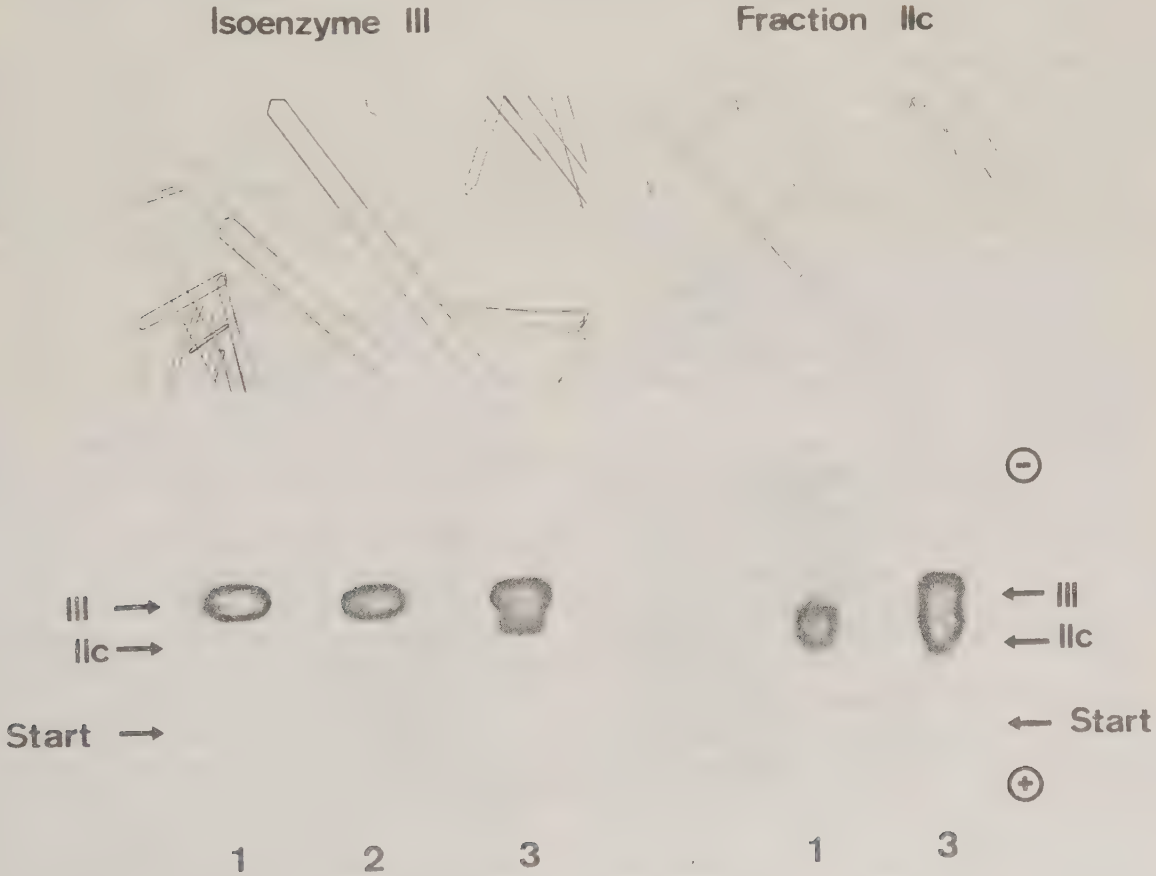

Fig. 3. Crystallization of horse liver ADH. a: Isoenzyme III; b: Fraction IIc. Crystals were collected from 5% (v/v) methanol in phosphate buffer. Electrophoresis on starch gel at pH 8.5, Tris-HCl buffer: (1) chromatographically purified fraction; (2) redissolved crystals; (3) supernatant.

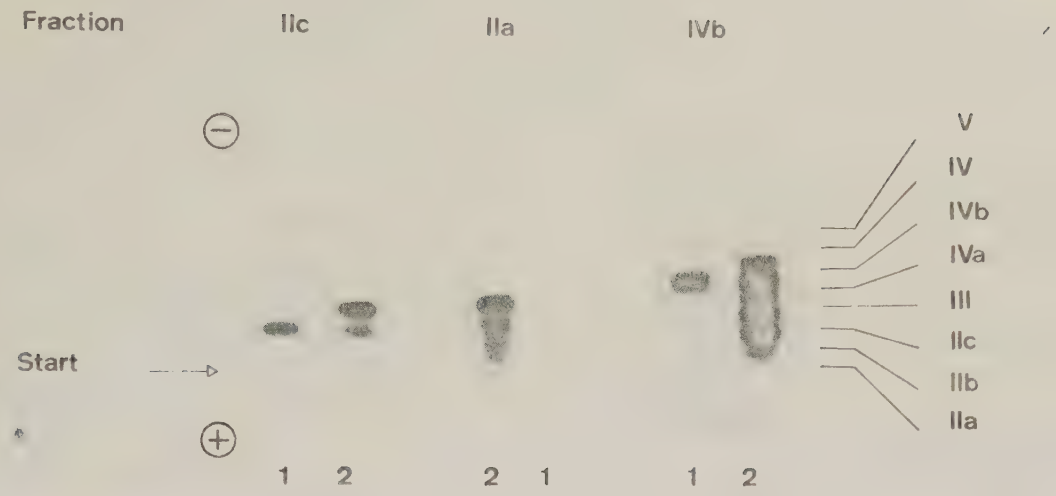

Fig. 4. Monomerization and reactivation of horse liver ADH. a: Fraction IIc; b: Fraction IIa; c: Fraction IVb. Electrophoresis on starch gel at pH 8.5, Tris-HCl buffer: (1) before monomerization and (2) after reactivation.

zyme III, upon standing in solution, forms fractions IIb and IIa in addition to fraction IIc, indicating a further interrelation between these fractions. In agreement with this observation monomerization and reactivation of fraction IIa yields a mixture of fractions IIa, IIb, IIc and isoenzyme III (fig. 4b). The existence of more than three isoenzymes (III, IV and V) could be explained in several ways:

(1) By the assumption of further subunits differing in primary structure from subunit A and B.

(2) By processes involving the cleavage of covalent bonds (disulfide bridge, peptide or acid amide). The full interconvertibility between the fractions IIa, IIb, IIc and isoenzyme III, however, excludes these two possibilities.

(3) The changes in charge underlying the differences in electrophoretic mobility and chromatographic properties of these interconvertible fractions could result from a reversible attachment of ligands as reported for drosophila ADH [15]. Since the interconversion occurs in the absence of free ligands such as zinc and coenzyme, this possibility seems unlikely. Furthermore, identical electrophoretic patterns were obtained in presence or absence of zinc, EDTA or coenzyme during monomerization and/or reactivation.

The concept of conformers advanced by Kaplan [16] offers an alternative explanation. The polypeptide of subunit A could adopt more than one conformation thus leading to subunits with the same primary structure but differing in physical-chemical properties. The existence of one alternate conformation (A′) would give rise to two conformers (AA′ and A′A′). Since there are more than two fractions interconverting with isoenzyme III (IIc, b and a) two conformationally different subunits (A′ and A″) are suggested. The question arises whether A′ and/or A″ can form enzymatically active dimers with the B subunit. As shown in fig. 4c, the reversible inactivation of the fraction IVb yields isoenzyme V, IV and III as well as the fractions IVb, IVa, IIc, IIb and IIa. This finding suggests that the fraction IVb represents a hybrid dimer between one of the conformationally changed A subunits and B.

The results show two different processes to be responsible for the multiple molecular forms of horse liver alcohol dehydrogenase:

(1) Isoenzymes III, IV and V are formed by random combination of two subunits A and B differing in primary structure.

(2) Three conformations of the A polypeptide (A, A′ and A″) seem to exist. They are sufficiently stable thermodynamically to form catalytically active dimers *in vitro* as well as in the liver cell. A study of the primary structure of the subunits of these conformers is in progress in order to corroborate this hypothesis.

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Heterogeneity of Horse Liver Alcohol Dehydrogenase

Purification and Characterization of Multiple Molecular Forms

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1. Twelve enzymatically active fractions of horse liver alcohol dehydrogenase are demonstrated by starch gel electrophoresis and chromatography on CM- and DEAE-cellulose. A method for the isolation and purification of seven of these multiple molecular forms is described. Their subunit composition is discussed.

2. The isoelectric point was determined for five of the enzymes by the method of isoelectric focusing.

3. Catalytic properties of isoenzyme III (subunit composition AA) are compared with those of isoenzyme V (BB) and enzyme IIc (AA′). Differences are observed with regard to the oxidation of alcohols, the reduction of aldehydes and the pH rate profiles.

A heterogeneity of alcohol dehydrogenase has been observed in various organisms, the number of molecular forms depending on the species [1—4]. Among these alcohol dehydrogenases isolated from different sources, the horse liver enzyme shows the highest degree of complexity. In crude horse liver extracts, five fractions were detected upon electrophoresis on agar and starch gel [5—7]. Improvement of the methods for electrophoretic and chromatographic separation has ascertained the existence of up to nine enzyme fractions [8,9]. The comparison recently made by Theorell [10] between the nine fractions described by Pietruszko *et al.* [9] and the nine fractions reported from our laboratory [11] indicates that only seven of these multiple molecular forms are identical. These results suggest that a total of at least eleven enzymatic fractions exist. The present communication demonstrates twelve enzymes, and reports on the purification and separation of seven of them.

We have previously reported on the subunit composition and physical-chemical properties of the multiple molecular forms of horse liver alcohol dehydrogenase [8,11]. Two different mechanisms are thought to be responsible for this heterogeneity: (a) three dimeric isoenzymes result from the combination of two subunits A and B [8,9] which have been shown to differ in primary structure [12]. (b) Interconversions *in vitro* between several enzyme forms suggest that the subunits exist in more than one conformation (*e.g.* A′ or B′), thus giving rise to additional enzyme forms (conformers) [11].

Enzyme. Alcohol dehydrogenase or alcohol:NAD oxidoreductase (EC 1.1.1.1).

The extensive knowledge on the catalytic mechanism of alcohol dehydrogenase has essentially been gained with the main isoenzyme of horse liver (isoenzyme III). Only a little is known on the kinetics of the other enzymes. A first study has indicated that the differences between the single isoenzymes are larger than the ones observed between the fractions thought to represent conformers [8]. Therefore, some kinetic parameters of two isoenzymes and one conformer were investigated in order to evaluate the dependence of catalytic function on the primary structure of the subunit (A or B) and on a conformational change (A or A′), respectively.

MATERIAL AND METHODS

Materials

All chemicals were reagent grade and used without further purification. NAD^+ and NADH were obtained from Boehringer Mannheim GmbH (Mannheim), alcohols and aldehydes from Merck AG (Darmstadt). Aldehydes were freshly distilled before use. Acrylamide, *N*,*N*′-methylenebisacrylamide and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were purchased from Fluka AG (Buchs), coomassie brilliant blue R 250 from Colab Laboratories (Chicago). Ion exchange celluloses were products of Serva Entwicklungslabor (Heidelberg) and of Whatman, W. & R. Balston (Maidstone). Ampholytes were obtained from LKB Produkter AB (Stockholm). Deionized water was used for the purification steps, metal free distilled water for analytical procedures.

Enzyme Assay

Alcohol dehydrogenase activity was assayed by measuring and recording the change in NADH concentration at 366 or 340 nm and 25° (Eppendorf Spectrophotometer or Gilford 222-T). The standard reaction mixture contained 33 mM sodium pyrophosphate buffer pH 8.8, 1.6 mM NAD^+ and 16 mM ethanol. For measurements of the reverse reaction, the following assay conditions were used: 33 mM sodium phosphate buffer pH 7.0, 0.33 mM NADH and 83 mM acetaldehyde. Enzyme activity was expressed in International Units (IU = μmoles substrate used per minute).

Protein Concentration

This was estimated by the method of Waddel [13]; a Gilford 222-T Spectrophotometer served to determine the absorbance at 215 and 225 nm. Absorbance at 280 nm was measured for purified isoenzyme fractions, and an extinction coefficient at 280 nm of 0.455 per cm for a 1 mg/ml solution of enzyme IIc, III, IV and V [14] was assumed.

Concentration of Enzyme Solutions

Enzyme solutions were concentrated with a filter apparatus SM 16243 with perbunan layer and ultrafilters SM 12136 (Sartorius Membranfilter GmbH, Göttingen) under nitrogen.

Electrophoresis

Electrophoresis on starch gel was carried out as described previously [11]. Disc electrophoresis was performed to assess relative purity of enzyme preparations. The method of Ornstein and Davis [15] was adapted to the properties of alcohol dehydrogenase. 4% polyacrylamide gels in 10 mM piperazine-HCl buffer pH 7.0 were prepared by polymerization at 40° for 2 h. The slight extra charge of the enzyme results in slow migration and does not allow higher gel concentration. Persulfate and unpolymerized acrylamide inhibit alcohol dehydrogenase probably due to modification of essential sulfhydryl groups. Therefore, gels were washed electrophoretically for 1 h with 2 mA per gel to remove persulfate. Subsequently, thioglycolic acid served to eliminate unpolymerized acrylamide: 0.2 ml of a 0.1 M solution of thioglycolic acid, adjusted to pH 7.0 with solid piperazine, was applied to each gel and preelectrophoresis was carried out over night with 1 mA per gel. 10 mM piperazin-HCl buffer pH 7.0 was used as a vessel buffer. Enzyme solutions were dialyzed against the same piperazine buffer prior to application. After renewal of the vessel buffer electrophoresis was carried out for 1.5—2 h at 4° with 2 mA per gel and 120 V; the anode was on top of the gels. The gels were stained for alcohol dehydrogenase activity as previously described [3] and for protein with coomassie brilliant blue [16].

Isoelectric Focusing

This was performed according to Vesterberg and Svensson [17] in a Uniphor Column Electrophoresis System (LKB Produkter AB, Stockholm) with pH gradients of pH 7—10. The enzyme fractions were dialyzed over night against 100 volumes of a 1% glycine solution, pH 7.0 and applied to the column as 1 ml samples containing approximately 10 mg of enzyme protein. A constant voltage of 300 V was applied resulting in an initial current of 6—8 mA which dropped to 0.5—1 mA within 24 h indicating full stabilization of the system. The column was emptied after 48 h at a flow rate of 1 ml per minute, the absorbance at 280 nm was recorded with a Uvicord II photometer (LKB Produkter AB, Stockholm). 2 ml fractions were collected and checked for pH and alcohol dehydrogenase activity.

Purification Procedure

A schematic diagram of the procedure is shown in Fig. 1. The whole purification was carried out at 4°. All fractions were analyzed for alcohol dehydrogenase activity, protein concentration, pH and conductivity. The electrophoretic mobility of the enzymes was monitored by starch gel electrophoresis. In all chromatographic steps ion exchange cellulose was prepared according to Peterson and Sober [18], equilibrated with starting buffer and packed into a 2.4×60 cm column. The flow rate was 50 to 60 ml per hour, and 10 ml fractions were collected.

Step 1: Extraction. Approximately 1 kg of fresh horse liver was ground in a meat chopper and extracted with the same volume (v/w) of sodium phosphate buffer pH 7.0, 10 mM for 3 h under continuous stirring followed by filtration through gauze. The filtrate was centrifuged at $23000 \times g$ for 4 h (MSE 18 high speed centrifuge) and the lipid layer was carefully removed before collection of the supernatant (fraction 1). The extract was dialyzed for three days against four times the ten-fold volume of 5 mM sodium phosphate buffer pH 6.8, and centrifuged for 4 h at $23000 \times g$. The supernatant (fraction 2) was used for further purification.

Step 2: First Fractionation of Enzymes. A separation of the enzyme fractions into two groups was achieved by column chromatography on CM-cellulose (Serva). Under starting conditions (5 mM sodium phosphate buffer pH 6.8) the enzymes I, II, IIa, IIb and IIc were eluted together with 85—90% of the total protein (fraction 3.1). Isoenzymes III, IV and V were adsorbed at the top third of the column. After washing with starting buffer isoenzymes III, IV and V were eluted together with hemoglobin in

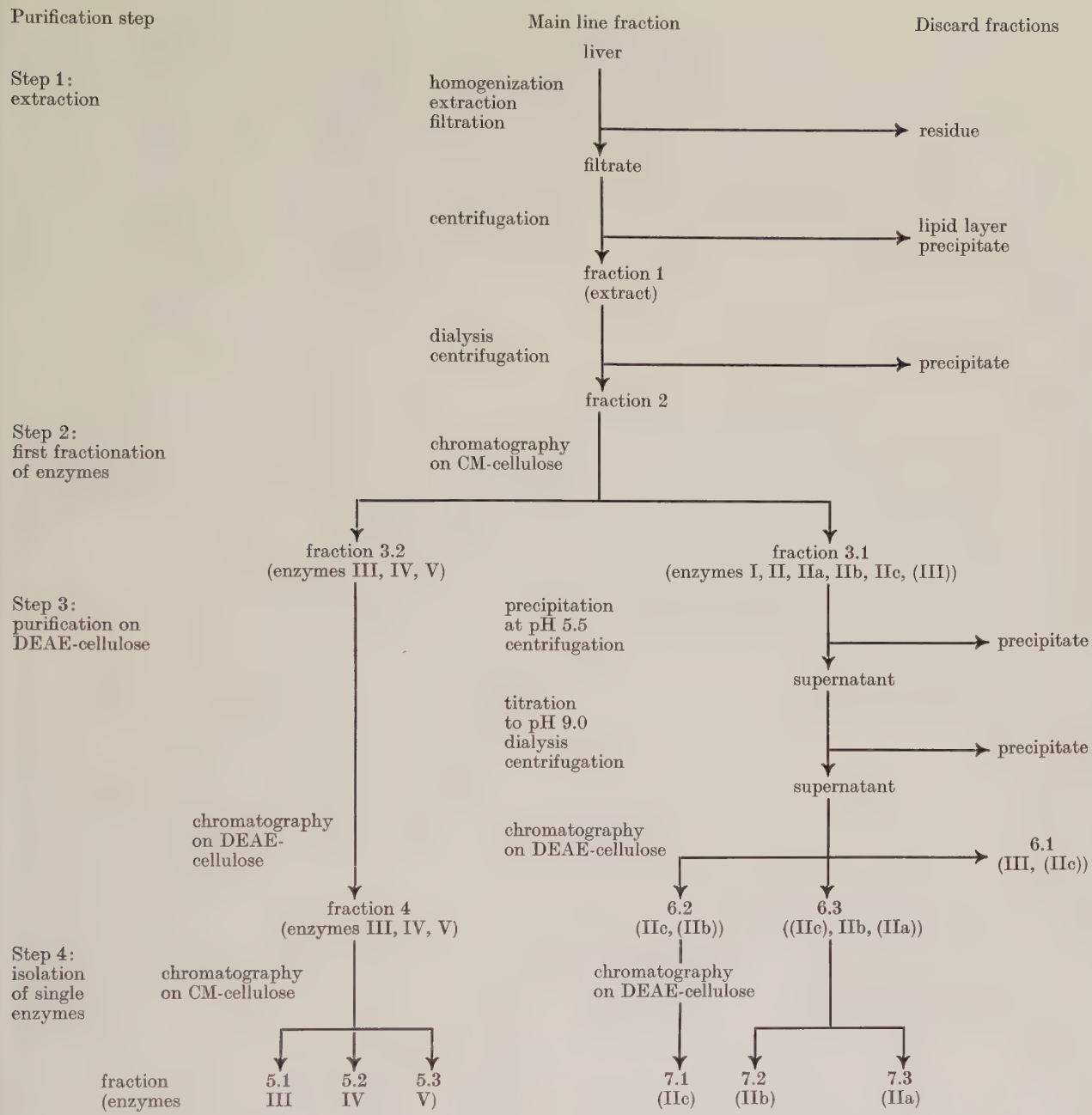


Fig. 1. *Purification of multiple molecular forms of horse liver alcohol dehydrogenase (flow sheet)*

one step with sodium phosphate buffer pH 7.0, 100 mM (fraction 3.2). At this stage of purification, the enzymatic activity of fraction 3.1 was stable allowing storage at 4° for several days, whereas the purification of fraction 3.2 had to be carried on without delay.

Step 3: Purification of the Enzymes on DEAE-Cellulose. Fraction 3.2 was dialyzed against three times the ten-fold volume of 10 mM Tris-Cl buffer pH 8.5 and applied to a column of DEAE-cellulose (Serva). The column was washed with equilibration buffer to complete the elution of the enzymes, which were not adsorbed under these conditions (fraction 4).

Fraction 3.1 was titrated to pH 5.5 with concentrated phosphoric acid and allowed to stand for 60 min at 4°. The precipitated protein was removed by centrifugation for 1 h at 23000 $\times g$. After adjust-

ment of the pH to 9.0 with solid Tris base and extensive dialysis against 10 mM Tris-Cl buffer pH 9.0 centrifugation was repeated. A column of Whatman DEAE-cellulose was used to chromatograph the supernatant. Under the starting conditions, all enzymatic activity and contaminating protein was adsorbed. The equilibration of the column with a 10 mM solution of lysine-hydrochloride, adjusted to pH 9.0 with solid Tris base, resulted in the elution of three peaks of alcohol dehydrogenase activity. The single peaks were enriched in the enzyme III, IIc and IIb, respectively although none of them was homogeneous upon starch gel electrophoresis. The peaks were pooled to fractions 6.1, 6.2, and 6.3, respectively. Fraction 6.1 containing mainly isoenzyme III was discarded.

Step 4: Isolation of Single Enzymes. The chromatographic separation of isoenzymes III, IV and V (in fraction 4) was achieved on CM-cellulose, that of the enzymes IIa, IIb and IIc (in fraction 6.2 and 6.3) on DEAE-cellulose.

Fraction 4 was dialyzed against three times the 100-fold volume of starting buffer in 36 h and run into a column of CM-cellulose (Whatman) equilibrated with 10 mM sodium phosphate pH 6.5. Part of the contaminating protein was washed out with the starting buffer. The adsorbed enzymes were eluted stepwise: isoenzyme III with 10 mM sodium phosphate buffer pH 7.0 (fraction 5.1), isoenzyme IV at pH 7.5 (fraction 5.2), isoenzyme V with a linear gradient from 10 to 50 mM sodium phosphate buffer, pH 7.5 (fraction 5.3).

Fractions 6.2 and 6.3 were rechromatographed on Whatman DEAE-cellulose columns under the same conditions described for fraction 3.1. The elution of fraction 6.2 with a solution of 10 mM lysine-hydrochloride pH 9.0 separated enzyme IIc from contaminating III and IIb. Enzymes IIb and IIa were collected from the analogous chromatography of fraction 6.3. The chromatographic fractions containing electrophoretically pure enzyme IIc, IIb or IIa were pooled to fraction 7.1, 7.2 and 7.3, respectively.

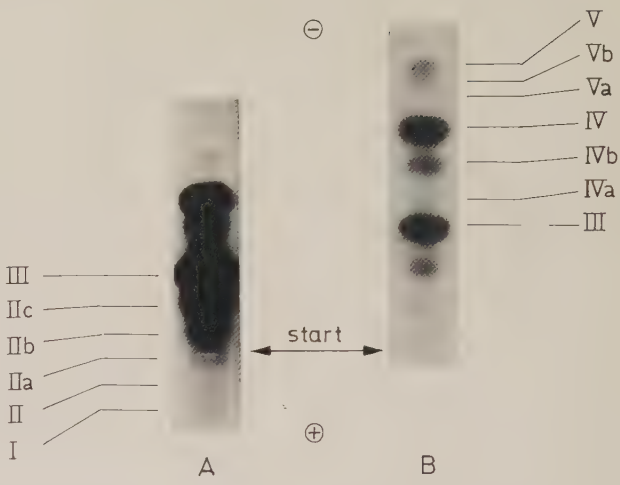


Fig. 2. *Starch gel electrophoresis of horse liver extract.* Gel buffer: 25 mM Tris-Cl, pH 8.5; vessel buffer: 300 mM Tris-Cl, pH 8.5; 160 V and 16—20 mA for 5 h (A) or 120 V and 10—12 mA for 14 h (B). Gels were stained for alcohol dehydrogenase activity with ethanol as substrate

Table. *Activity of fractions attending purification*

Fraction	Alcohol dehydrogenase activity		Specific activity	Total protein	Yield
	per ml	total			
	IU/ml	IU	IU/mg	g	%
1. Extract	9.9	8110	0.19	42.5	100
2. After dialysis	8.74	6680	0.20	32.9	82.5
3.1. CM-cellulose	0.98	687	0.02	29.0	8.4
acid treated	0.98	678	0.03	21.2	8.4
3.2. CM-cellulose	25.6	3690	0.82	4.5	45.5
4. III, IV, V	18.4	3680	1.58	2.3	45.4
5.1. III	10.59	1207	4.66	0.258	14.9
5.2. IV	6.04	440	2.58	0.170	5.4
5.3. V	0.46	18	0.23	0.079	0.2
6.2. IIc, (IIb)	1.34	147	1.38	0.118	1.8
6.3. (IIc), IIb, (IIa)	0.67	228	1.40	0.163	2.8
7.1. IIc	0.18	66	2.86	0.023	0.8
7.2[a]. IIb	0.21	18	1.32	0.015	—
7.3[a]. IIa	0.16	8	1.28	0.006	—

[a] Isolated from a different extract.

RESULTS AND DISCUSSION

Isolation and Purification of Single Enzymes

The Table summarizes the results of a typical isolation of the multiple molecular forms of alcohol dehydrogenase according to the fractionation procedure outlined in the flow sheet (Fig. 1). Extracts from individual horse livers show a considerable variability with respect to alcohol dehydrogenase activity. The total activity extracted from 1 kg of fresh liver (fraction 1) may range from 5000 to 10000 IU. The heterogeneity of the alcohol dehydrogenase in the extract can be demonstrated by electrophoretic separation on starch gel and subsequent activity staining (Fig. 2A and B). According to their electrophoretic mobility the multiple molecular forms have been denominated I, II, IIa, IIb, IIc, III, IVa, IVb, IV and V, enzyme V showing the fastest migration towards the cathode. Nine bands are observed in all extracts. The existence of a tenth band (enzyme II, Fig. 2A), appearing in some extracts, can be ascertained by electrophoretic analysis of chromatographically enriched fractions (Fig. 3P).

A variance in the contribution of the single enzymes to the total activity is observed. However, enzyme III always represents the main fraction when electropherograms are evaluated by densitometry.

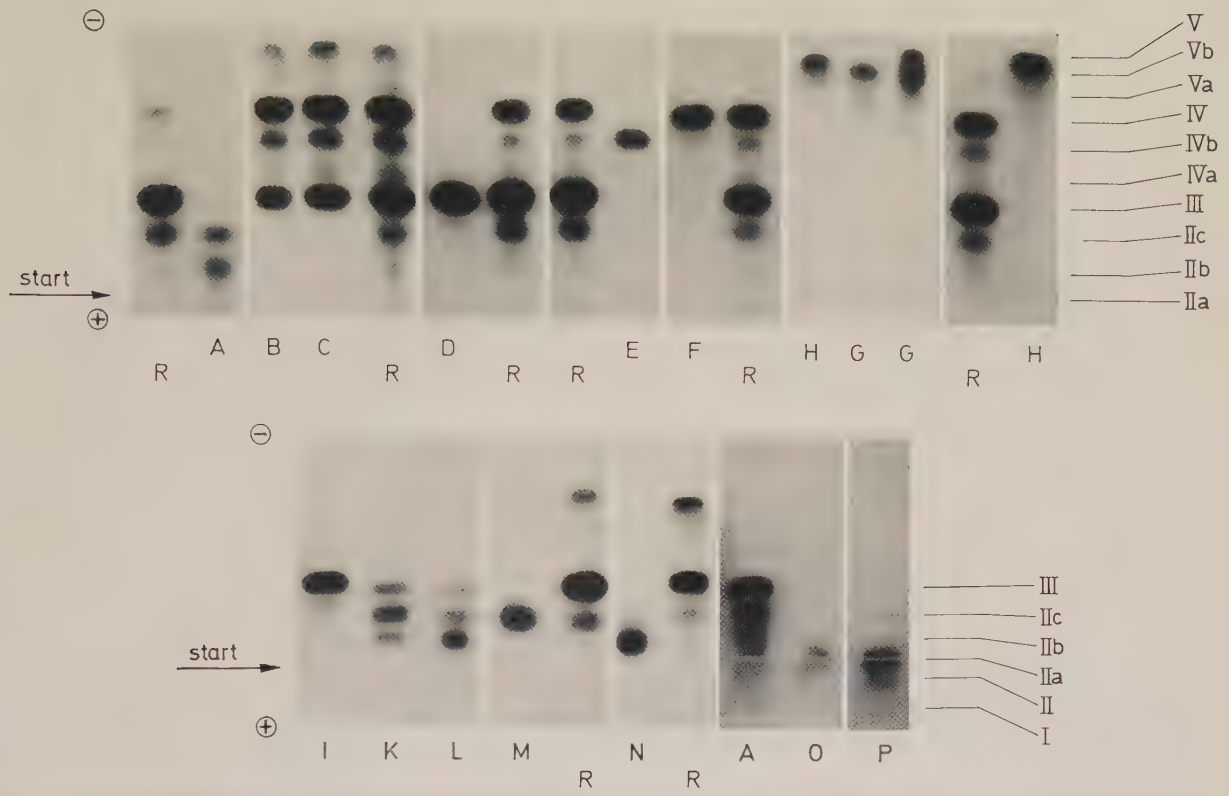


Fig. 3. *Starch gel electrophoresis of fractions attending purification of horse liver alcohol dehydrogenase.* For methods see Fig. 2 B. (A) Fraction 3.1; (B) fraction 3.2; (C) fraction 4; (D) isoenzyme III; (E) enzyme IVb; (F) isoenzyme IV; (G) fractions enriched in enzymes Va and Vb; (H) isoenzyme V; (I) fraction 6.1; (K) fraction 6.2; (L) fraction 6.3; (M) enzyme IIc; (N) enzyme IIb; (O) enzyme IIa; (P) fraction enriched in enzyme II; (R) extract as a reference

It is followed by enzyme IV, whereas enzyme V contributes only a few per cent to the total activity and shows the largest individual variability. These three enzymes are formed by the possible dimeric combinations of two subunits A and B; they represent isoenzymes with the subunit composition AA (isoenzyme III), AB (isoenzyme IV) and BB (isoenzyme V) [11, 19]. Jörnvall has recently shown the two subunits to differ in amino acid sequence [12].

The other enzyme fractions observed in the extract (Fig. 2 A and B) are thought to be conformers. We have previously described a full interconversion between the enzymes IIc, IIb and IIa and isoenzyme III, suggesting the exsistence of three conformations of the polypeptide A (A, A′ and A″) [11]. A subunit composition A′B and A″B was attributed to the enzyme IVb and IVa, respectively. In livers containing relatively large amounts of isoenzyme V some activity is observed between the electrophoretic bands of isoenzyme IV and V. After chromatographic enrichment two additional enzymes (Va and Vb) can clearly be demonstrated on starch gel (Fig. 3 G). This finding indicates that the polypeptide B also exists in two conformations (B and B′). Although the single conformers are minor fractions, their activity sums up to a considerable part (20 to 40%) of the total activity in the extract. Fractionation procedures such as precipitation by organic solvents, ammonium sulfate or heat, previously used for the purification of horse liver alcohol dehydrogenase, have been omitted in order to avoid possible changes in the conformation of the multiple molecular forms. The purification and isolation is based exclusively on chromatography on ion exchange cellulose. During the dialysis of the extract against the starting buffer of the first chromatography 10 g of protein and some activity are lost (Table). However the enzyme pattern, as determined by electrophoresis, remains unchanged. The chromatography on CM-cellulose brings about a first fractionation of alcohol dehydrogenase (Fig. 4). Enzymes I, II, IIa, IIb and IIc are eluted in a dense, brown solution containing most of the protein as indicated by the drop in specific activity (fraction 3.1); enzymes III to V are adsorbed under starting conditions and eluted together with hemoglobin by increasing the pH and conductivity of the phosphate buffer (fraction 3.2). A 4-fold purification of these enzymes results from this step. The yield of the chromatography is 60—70% (Table). The electropherograms of fraction 3.1 and

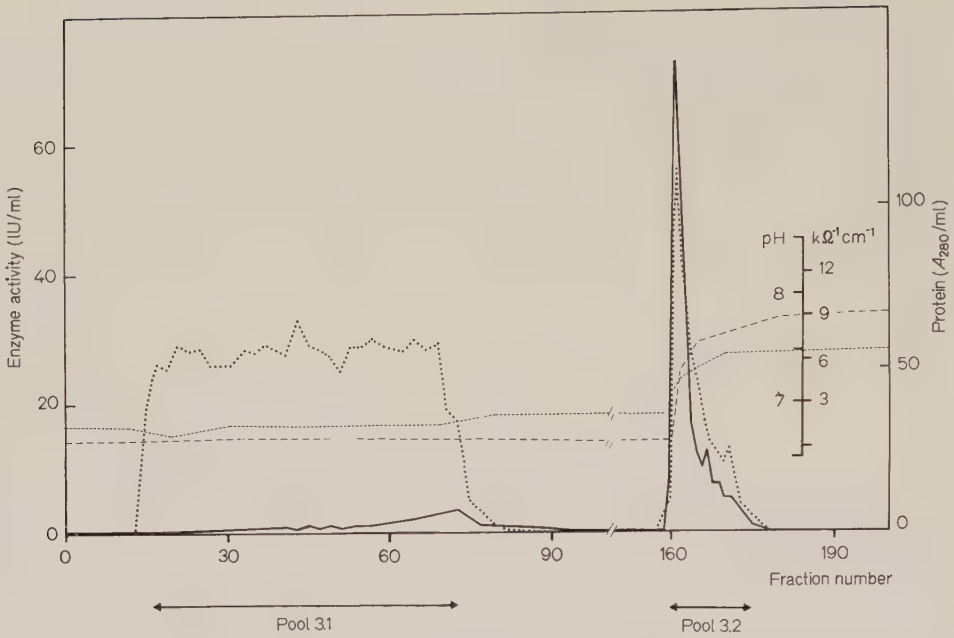


Fig. 4. *First fractionation of horse liver alcohol dehydrogenase on CM-cellulose.* Elution of enzymes I, IIa, IIb, IIc (fraction 3.1) with starting buffer, 5 mM sodium phosphate, pH 6.8. Elution of enzymes III, IVa, IVb, IV, Va, Vb, and V (fraction 3.2) with 100 mM sodium phosphate, pH 7.5. 9 ml fractions were collected. ——, alcohol dehydrogenase activity (IU/ml); ·····, protein (absorbance at 280 nm/ml); ------, pH; —·—·—, conductivity ($k\Omega^{-1}$ cm^{-1})

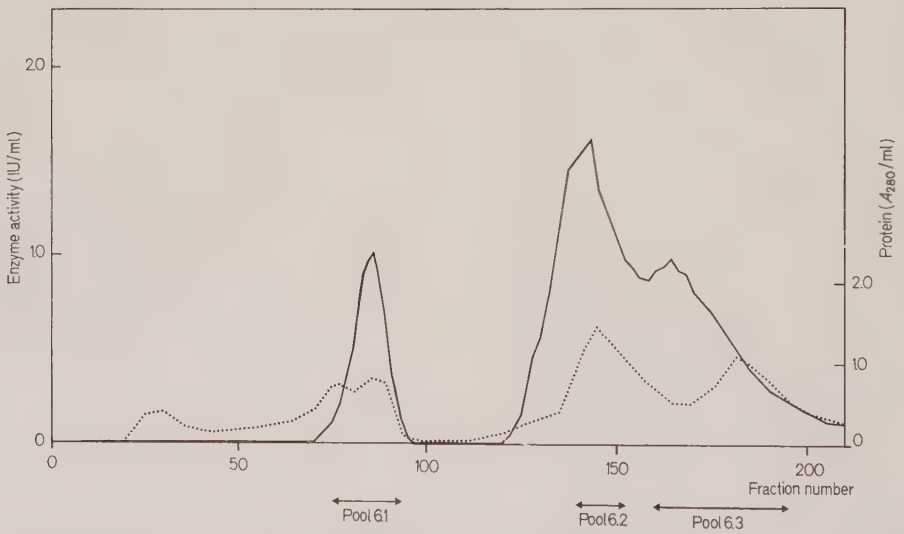


Fig. 5. *Purification of fraction 3.1 on DEAE-cellulose.* Starting buffer: 10 mM Tris-Cl, pH 9.0. Elution with 10 mM lysine hydrochloride, pH 9.0 was initiated at fraction 1. 9 ml fractions were collected. Fraction 6.1: mainly enzyme III; fraction 6.2: mainly enzyme IIc; fraction 6.3: mainly enzyme IIb. ——, alcohol dehydrogenase activity (IU/ml); ·····, protein (absorbance at 280 nm/ml)

3.2 are shown in Fig. 3A and B. A substantial purification of the fractions 3.1 and 3.2 proved necessary before a proper isolation of the single enzymes could be achieved. Therefore, both fractions are submitted to chromatography on DEAE-cellulose in step 3. This chromatography separates isoenzyme III, IV and V in fraction 3.2 from hemoglobin with a step recovery of nearly 100%; the isoenzyme pattern in the colourless fraction 4 remains unchanged (Fig. 3C). Fraction 3.1 contains most of the protein extracted from liver tissue amounting to a total of 30 to 40 g. Approximately 30% of the impurities can

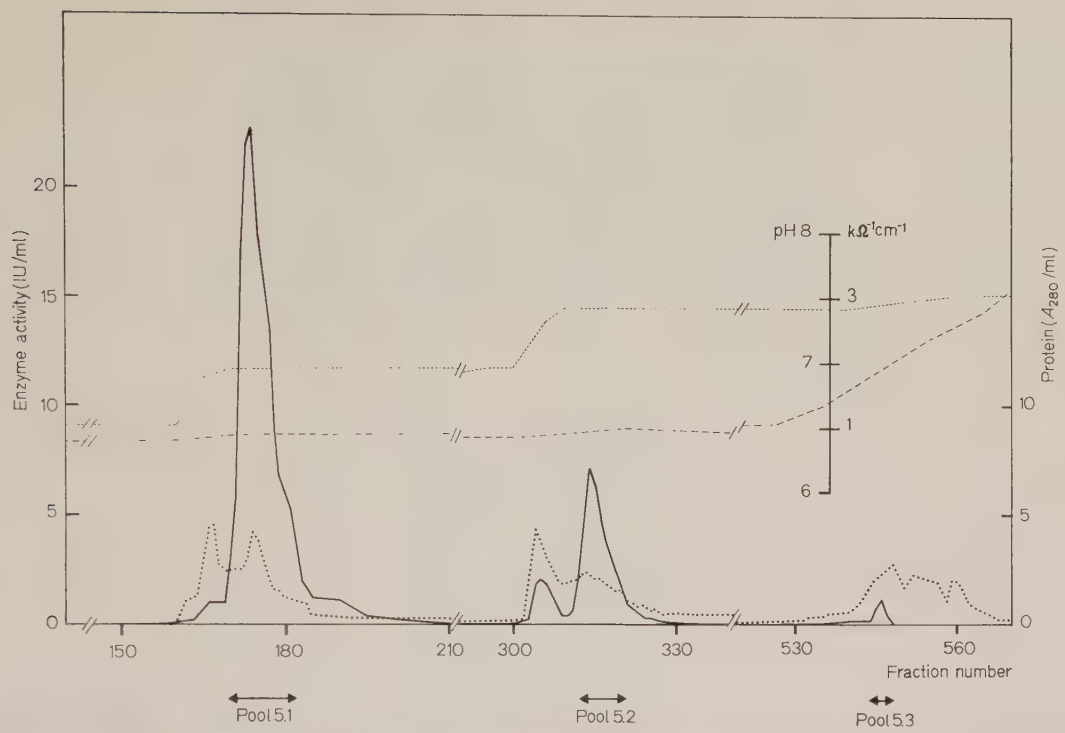


Fig. 6. *Isolation of single enzymes on CM-cellulose.* Stepwise elution of isoenzyme III (fraction 5.1) with 10 mM sodium phosphate, pH 7.0, isoenzyme IV (fraction 5.2) with 10 mM sodium phosphate, pH 7.5, and isoenzyme V (fraction 5.3) with a linear gradient from 10 to 50 mM sodium phosphate, pH 7.5. 9 ml fractions were collected. ——, alcohol dehydrogenase activity; ·····, protein (absorbance at 280 nm/ml); ------, pH; —·—·—, conductivity ($k\Omega^{-1}$ cm^{-1})

be removed by an acid precipitation without loss of enzymatic activity. This pretreatment prevents clogging of the column and facilitates the elution of alcohol dehydrogenase from DEAE-cellulose with lysine. Alcohol dehydrogenases are basic proteins due to their high content in lysine and arginine [14], and are near their isoelectric point at pH 9.0, the pH of the starting buffer. Small concentrations of lysine are sufficient to elute the enzymes specifically. This suggests that they are bound to the cellulose by hydrogen bonds between the unprotonated diethylaminoethyl groups of the cellulose and the free amino groups of basic amino acid residues. The contaminating proteins, adsorbed to the cellulose by electrostatic forces are not eluted with lysine, resulting in a 50- to 80-fold purification of the enzymes. The elution pattern of a typical chromatography is shown in Fig. 5. The first peak (fraction 6.1) contains mainly isoenzyme III and some enzyme IIc (Fig. 3. I) and is discarded. The fractions of the second and third peak are analyzed by starch gel electrophoresis. They are pooled according to their enzyme composition in order to obtain an enrichment of enzyme IIc in fraction 6.2 and of the enzymes IIb and IIa in fraction 6.3 (Fig. 3 K and L).

The final step (step 4) brings about the chromatographic isolation of the single enzymes. Pure enzymes IIc, IIb and IIa are obtained by separate rechromatography of fraction 6.2 and 6.3 on Whatman DEAE-celllulose. The main peak from the elution of fraction 6.2 represents pure enzyme IIc (fraction 7.1 in Fig. 3 M). Two adjacent peaks mainly contain isoenzyme III and enzyme IIb and are discarded. Only small amounts of pure enzymes IIb and IIa (fractions 7.2 and 7.3 in Fig. 3 N and O) are obtained by an analogous chromatography of fraction 6.3 because of contamination with adjacent enzymes. The individual variability of the enzyme pattern does not allow an isolation of enzyme IIb and IIa from each extract. The enzymes I and II have not yet been obtained in electrophoretically pure form.

Fig. 6 illustrates the stepwise elution of the pure isoenzymes III (fraction 5.1), IV (fraction 5.2) and V (fraction 5.3) from CM-cellulose. Although enzyme IVb is eluted as a distinct peak prior to isoenzyme IV, it is often contaminated with adjacent bands. Enzyme IVa trails after isoenzyme III. Enzymes Va and Vb, when present in the extract, appear as a shoulder before the peak of isoenzyme V. The electrophoretic mobility of all enzyme fractions remains unaffected by the purification procedure (Fig. 3 D to H).

At this stage of the purification isoenzyme III is 30 to 50% pure, isoenzyme IV and enzyme IIc

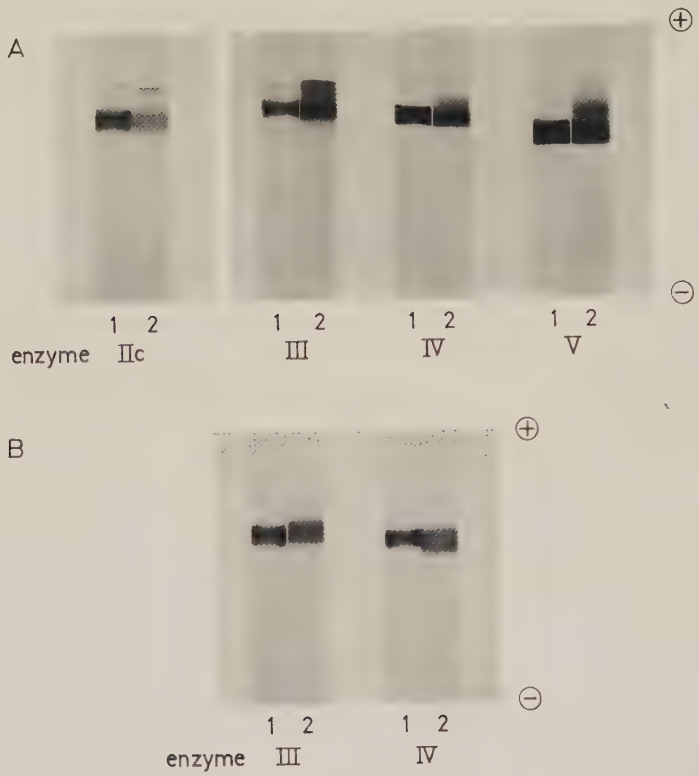


Fig. 7. *Disc electrophoresis of horse liver alcohol dehydrogenase.* 4% polyacrylamide gel; 10 mM piperazine-HCl buffer, pH 7.0; 120 V (2—3 mA per gel) for 90 to 120 min; gels were stained for alcohol dehydrogenase activity (1) and protein (2). (A) Enzymes IIc, III, IV and V after chromatographic purification; (B) isoenzymes III and IV after repeated crystallization

over 50%, while the purity of isoenzyme V depends on the amount originally present in the extract. The contaminating protein is shown by disc electrophoresis (Fig. 7 A). A final purification of isoenzyme III and IV is achieved by repeated crystallization from phosphate buffer containing 10 and 25% methanol, respectively. Homogeneous enzyme proteins are obtained (Fig. 7 B). The specific activity reaches a value of 9.5 IU/mg for isoenzyme III and 4.2 IU/mg for isoenzyme IV, when determined under standard assay conditions. The highest values so far reached for the specific activity of chromatographically purified isoenzyme V was 2.1 IU/mg and 5.0 IU/mg for crystalline enzyme IIc.

Isoenzyme III and the enzyme IIc convert to each other in methanol containing buffer of various pH. This interconversion can be prevented by storage of the enzymes in the crystalline state or by addition of 2-methyl-2,4-pentanediol to the enzyme solution. However, this alcohol, which has been used for the crystallization of alcohol dehydrogenase for X-ray analysis [20] seems to affect the catalytic properties of isoenzyme III within a few weeks. This phenomenon is being further investigated.

Isoelectric Points of Single Enzymes

The isoelectric points of isoenzyme III and IV and of the enzymes IIc, IIb and IIa were determined by means of isoelectric focusing. Characteristic pattern of the electrophoretic separation in an ampholyte gradient are shown in Fig. 8 A and B. Samples of isoenzyme III, IV and V from the last chromatography were mixed and applied to the column. Nearly 100% of the activity was regained after elution. The fully separated peaks (Fig. 8 A) were shown to represent pure isoenzymes by starch gel electrophoresis. An isoelectric point of 8.7 ± 0.01 was determined for isoenzyme III in three experiments proving a high reproducibility of the procedure. A value of 9.29 was obtained for isoenzyme IV. Isoenzyme V migrates into the cathode solution (pH > 10) not allowing an exact determination of its isoelectric point. An analogous experiment was carried out with fraction 6.3 giving an isoelectric point of 8.51, 8.28 and 8.08 for enzyme IIc, IIb and IIa, respectively (Fig. 8 B). A comparison of the isoelectric point shows that the differences between the values for isoenzyme III (AA), IV (AB) and V (BB) are larger than for the enzyme III, IIc, IIb

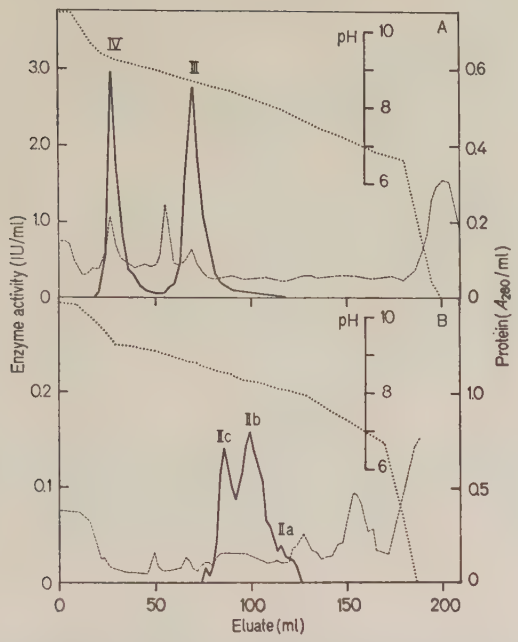


Fig. 8. *Isoelectric focusing of horse liver alcohol dehydrogenase.* Ampholyte gradients pH 7—10; 300 V for 48 h; 6 mA decreasing to 0.5 mA within the first 24 h. Elution rate: 1 ml per minute; 2 ml fractions were collected; enzymes were identified by starch gel electrophoresis. (A) Isoenzyme III and IV; (B) enzymes IIa, IIb and IIc. ——, alcohol dehydrogenase activity (IU/ml); ·····, pH; ------, protein (absorbance at 280 nm/ml)

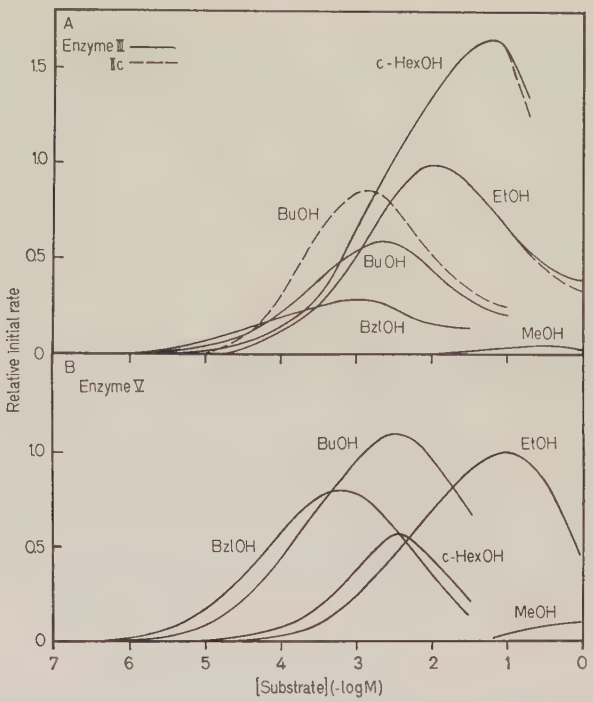


Fig. 9. *Oxidation of alcohols by single enzymes of horse liver alcohol dehydrogenase.* (A) Isoenzyme III (——) and enzyme IIc (– – – –); (B) isoenzyme V (——). Dependence of the relative initial rates on the substrate concentration; apparent V_{max} with ethanol it set equal to unity. MeOH, methanol; EtOH, ethanol; BuOH, butanol; c-Hex-OH, cyclohexanol; BzlOH, benzyl alcohol. Conditions: 33 mM sodium pyrophosphate buffer, pH 8.8, 1.6 mM NAD^+, 25°

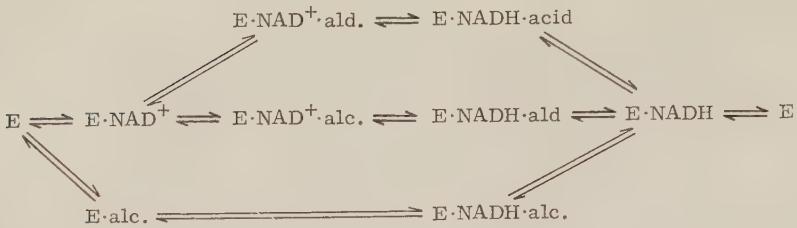
and IIa (constituted of the subunits A, A′ and A″). Six amino acid substitutions between the subunits A and B have been reported, which render the B subunit more positive by three charge units [12]. This observation is in accordance with the differences in isoelectric point between isoenzyme III, IV and V. The isoelectric points of enzyme III, IIc, IIb and IIa lie within 0.6 pH units, indicating that the conformational change from A to A′ and A″ induces only small shifts in the surface extracharge. Consequently these enzymes are poorly separable by electrophoresis and chromatography.

Catalytic Properties of Horse Liver Alcohol Dehydrogenase

Alcohol dehydrogenase of various species is known to exhibit a wide specificity for alcohols as well as for aldehydes and ketones [21—23, 1]. According to the Theorell-Chance mechanism [21] the last step of the reaction, that is the dissociation of the respective product coenzyme from the enzyme, limits the overall reaction velocity of horse liver alcohol dehydrogenase with primary alcohols and aldehydes [22]. Studies on the enzyme-nucleotide dissociation constants and on product inhibition have indicated an ordered mechanism including central and abortive ternary complexes (Scheme 1) [21, 22, 24]. Most of these studies have been carried out on commercial preparations known to contain isoenzyme IV and enzymes IIc, IIb and IIa besides isoenzyme III [7] or on crystalline enzyme known to contain at least enzyme IIc [25]. Homogeneous preparations of isoenzyme III become heterogenous upon storage by conversion to enzyme IIc, IIb and IIa [11, 10]. We have therefore checked all enzyme fractions for electrophoretic homogeneity before use.

Oxidation of Alcohols

Three primary alcohols (methanol, ethanol, and butanol), a cyclic (cyclohexanol) and an aromatic compound (benzyl alcohol) were tested as substrates. The dependence of the initial rates on the substrate concentration is shown in Fig. 9 A for the enzymes IIc and III and in Fig. 9 B for isoenzyme V. A comparison of the oxidation of ethanol by isoenzyme III and V shows mainly two differences. The



Scheme 1. E = alcohol dehydrogenase; alc. = alcohol; ald. = aldehyde

apparent Michaelis constants, as estimated from the Eadie-Hofstee plot ([S]/*v versus* [S]), for isoenzyme V is more than double the value for isoenzyme III. High substrate concentrations lead to substrate excess inhibition with both enzymes. For isoenzyme III this phenomenon seems to be due to the formation of the partially abortive ternary complex E. NADH. alc. (Scheme 1) [22]. Its slow dissociation to E. alc. and NADH prevents a full inhibition leaving a residual activity of about 40% at an ethanol concentration higher than 1 M. With isoenzyme V however, the E. NADH. alc. complex appears to be fully abortive. This difference between isoenzyme III and V holds true for all alcohols tested.

As predicted from the Theorell-Chance mechanism the enzymes IIc and V exhibit approximately the same V_{max} (estimated from the Eadie-Hofstee plot) with butanol as with ethanol. In contrast, the V_{max} of isoenzyme III with butanol only reaches about 50% of the value with ethanol. This deviation could be due to a change in the dissociation constants of E. alc. and/or E. NAD^+. alc. and/or in the rate of conversion of central ternary complexes (Scheme 1). Identical maximal rates with ethanol and butanol have been reported by Dalziel [22]. This divergence could be attributed to differences in experimental conditions such as pH or to the possibility that the older preparations of isoenzyme III were contaminated with considerable amounts of enzyme IIc, which does not show this phenomenon.

The most striking difference between isoenzyme III and V is found for clyclohexanol oxidation. Isoenzyme III is activated by intermediary concentrations of cyclohexanol resulting in an apparent V_{max} of 165% of the value obtained with ethanol, whereas the V_{max} extrapolated from the rates at low substrate concentrations are the same for both alcohols. High substrate concentrations finally lead to inhibition. In contrast to the other alcohols the E. NADH. alc. complex with cyclohexanol is not partially abortive but dissociates at a faster rate than the E. NADH complex, thus leading to activation; inhibition is effected by the direct formation of the binary complex from enzyme and substrate [26]. For isoenzyme V the ternary complex seems to be fully abortive also with cyclohexanol. An alternative explanation for the lack of substrate activation would be the formation of the binary complex E. alc. at relatively low substrate concentrations.

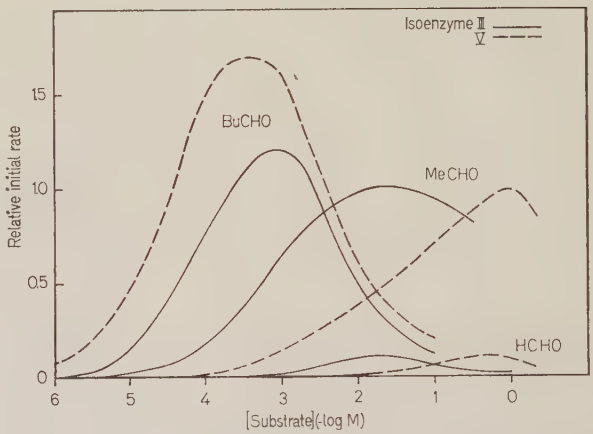


Fig. 10. *Reduction of aldehydes by horse liver alcohol dehydrogenase isoenzymes.* Isoenzyme III (——) and V (– – – –). Dependence of the relative initial rates on the substrate concentration; apparent V_{max} with acetaldehyde is set equal to unity. HCHO, formaldehyde; MeCHO, acetaldehyde; BuCHO, butyraldehyde. Conditions: 33 mM sodium phosphate buffer, pH 7.0, 0.33 mM NADH, 25°

It is noteworthy that the enzymes IIc and V exhibit the same broad substrate specificity known for isoenzyme III. The apparent Michaelis constants estimated for the various alcohols are very similar for the three enzymes. Relatively small differences are observed between isoenzyme III and enzyme IIc. The difference in primary structure between subunit A and B results in divergent kinetic behaviour of isoenzyme III and V with respect to deviations from the Theorell-Chance mechanism.

Reduction of Aldehydes

The results obtained with three aldehydes (Fig. 10) are in accordance with the observations made with the corresponding alcohols. The apparent K_m value of isoenzyme V with acetaldehyde is higher than the one of isoenzyme III. An increase of the chain length brings about a decrease of the Michaelis constants of butyraldehyde for both enzymes and the relative initial rates with formaldehyde are low.

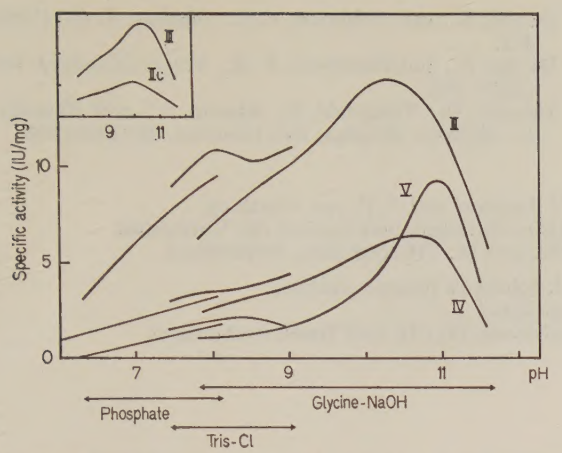


Fig. 11. *Dependence of the specific activity of single enzymes of horse liver alcohol dehydrogenase on the pH.* Determination of initial rates in presence of 16 mM ethanol, 1.6 mM NAD^+ and 33 mM buffer, 25°

In all cases the ternary complex E. NAD^+. alc. seems to be formed. This represents the initial step in the aldehyde dehydrogenase reaction of alcohol dehydrogenase (Scheme 1) [27]. Hence, NAD^+ is reduced, imposing as a substrate excess inhibition in the spectrophotometric test. High butyraldehyde concentrations lead to nearly complete inhibition, indicating that the rate of aldehyde dehydrogenase activity can match the value of the aldehyde reduction. With acetaldehyde the shape of the curves is broader and the values for the apparent V_{max} are lower than with butyraldehyde. Thus, the ternary complex E. NAD^+. ald. with acetaldehyde seems to be partially abortive.

Dependence of the Specific Activities on the pH

As shown in Fig. 11 marked differences appear between the pH rate profiles of the isoenzymes III, IV and V. The specific activity of isoenzyme III steadily increases with an elevation of the pH up to an optimum around pH 10.3. Enzyme IIc exhibits a similar curve but at lower specific activities. Under standard assay conditions (pyrophosphate buffer, pH 8.8) the specific activity of isoenzyme V is approximately one fourth of the value for isoenzyme III. Due to a different shape of the profile with a sharp optimum at pH 10.9, the specific activity of isoenzyme V reaches values close to the ones of isoenzyme III at high pH.

Isoenzyme IV does not show the intermediary profile expected under the assumption that the subunits A and B each contribute half the activity of the corresponding dimer to the hybrid enzyme AB. These results suggest that the mutual activation of the subunits depends on the partner in the dimer.

The differences in the catalytic properties between the isoenzymes III and V are more pronounced than between the enzymes IIc and III. The differences between the isoenzymes III and V with respect to the kinetic parameters outlayed in this communication and the steroid dehydrogenase activity [7, 8, 28] indicate that the amino acid substitutions between the subunits A and B do not affect the conformation of the protein only, but that some of these amino acid residues are involved in the active site of the enzyme. In this comparison of isoenzymes (III and V) on the one hand and conformers (IIc and III) on the other one has to take into account that enzyme IIc probably represents the dimer AA′. It is difficult to extrapolate to A′A′ which would be the analogous conformer to the isoenzyme BB.

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Lebenslauf

Am 17. November 1943 als Tochter des Otto und der Clara Lutstorf-Ledermann geboren, bin ich 1950 in die Primarschule eingetreten. Ich habe die Mittelschule und das Gymnasium in Bern besucht und im Herbst 1962 mit der Maturitätsprüfung abgeschlossen. Nach vierjährigem Studium der Mikrobiologie und Biochemie war ich während drei Semestern als Hilfsassistentin am Institut für allgemeine Mikrobiologie in Bern tätig. Während dieser Zeit habe ich mich mit der Alkoholdehydrogenase der Hefe beschäftigt, um im Wintersemester 1967/68 das Diplomexamen mit Erfolg zu bestehen. Im Februar 1968 habe ich unter der Leitung von Prof. Dr. J.P. von Wartburg mit der Durchführung meiner Dissertation begonnen. Gleichzeitig war ich als Assistentin am Medizinisch-chemischen Institut beschäftigt.